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(54) Title: α -HELICAL PEPTIDE NUCLEIC ACID α -PNA

(57) Abstract: The present invention relates to peptide-based nucleic acid surrogates (PNAs) having a repeating structure of $(AA^B-aa_n)_m$ and a particular secondary structure that can bind to particular single-stranded nucleic acid targets. Preferably the peptide-based nucleic acid surrogate has an alpha-helical secondary structure (α PNA). Also, the present invention relates to the method of forming peptide-based nucleic acid surrogates having a particular secondary structure. The nucleic acid surrogates may be utilized for therapeutic (antisense, antigene), diagnostic (genetic), and molecular switching (α PNA chips) applications.

α -HELICAL PEPTIDE NUCLEIC ACID (α PNA)

Field of the Invention

5 The present invention relates to the design, synthesis, and potential applications of α -helical peptide nucleic acids (α PNAs) which are new molecular constructs that merge the molecular recognition elements of nucleic acids with the predictable structural features of an α -helical peptide scaffold. These new peptide-based nucleic acid surrogates bind with high affinity and
10 specificity to complementary single stranded DNA and are capable of binding to both single stranded RNA and double stranded DNA targets. The present invention is directed to the use of α PNAs for therapeutic (antisense, antigene), diagnostic (genetic), and molecular switching (α PNA chip) applications.

Background of the Invention

15 In most DNA-regulatory proteins, α -helical subunits serve as the molecular scaffolding for presentation of key amino acid side chains to their specific nucleic acid binding sites. (Cf. Steitz, T.A. Q. Rev. Biophys. 1990, 23, 205). Sequence-specific binding of these α -helical binding domains to double-
20 stranded DNA occurs in the major groove as a consequence of multiple interactions arrived at (combinatorially) through evolutionary selection. Despite work toward the elucidation of Nature's "code" for molecular recognition of double-stranded DNA, it is not yet feasible to rationally design a peptide structure that will bind to any particular DNA duplex. (Desjarlais, J.R., Ber, J.M. Proc. Natl.
25 Acad. Sci. U.S.A. 1992, 89, 7345; Desjarlais, J.R., Berg, J.M. Proc. Natl. Acad. Sci. U.S.A. 1993, 90, 2256; Rebar, E.J., Pabo, C.O. Science 1994, 263, 671; Jamieson, A.C., Kim, S.-H., Wells, J. A. Biochemistry 1994, 33, 5689; Choo, Y., Klug, A. Proc. Natl. Acad. Sci. U.S.A. 1994, 91, 11163; Segal, D.J., Dreier, B., Beerli, R. R., Banbus, C. F. III; Proc. Natl. Acad. Sci. U.S.A. 1999, Vol. 96,

2758). On the other hand, molecular recognition of single-stranded nucleic acids via Watson-Crick (W-C) base pairing is quite predictable and provides the basis for antisense approaches to genetic manipulation. (Antisense Research and Application; Crooke, S. T.; Springer-Verlag: Berlin, 1998).

5 The pioneering discovery that the (2-deoxy)ribose phosphate framework of nucleic acids could be replaced by a much simpler polyamide backbone has spawned considerable interest in "peptide nucleic acids" (PNAs) and related constructs. (Dueholm, K. L., Nielsen, P.E. New J. Chem. 1997, 21, 19; Eriksson, M., Nielsen, P.E., Q. Rev. Biophys. 1996, 29, 369). However, the
10 merger of an α -helical peptidyl scaffold with well-defined nucleobase molecular recognition patterns (W-C base pairing for single-stranded RNA and Hoogsteen base pairing for double-stranded DNA) has not been explored. A particular advantage of such hybrid structures, termed as an α -helical peptide-based nucleic acid or α PNA, give the opportunity to functionalize the peptide scaffold
15 itself to enhance transport, hybridization, and other properties.

Summary of the Invention

The present invention relates to the design, synthesis, and potential applications of α -helical peptide nucleic acids (α PNAs), which are new
20 constructs that merge the molecular recognition elements of nucleic acids with the predictable structural features of an α -helical peptide scaffold. The described α PNAs bind with high affinity and specificity to complementary single-stranded DNA and are capable of binding to both single-stranded RNA and double-stranded DNA targets. Although there are examples of peptide based nucleic
25 acid surrogates, the use of peptide secondary structure to achieve the nucleobase spacing required for base-pairing to helical nucleic acids is conceptually novel.

The α PNA platform has a major advantage over known art in that it allows for the ready incorporation of functionality to enhance binding (affinity,

kinetics, specificity) and modify/introduce other properties (solubility, transport, stability). Also, multifunctional α PNAs may be designed for use as artificial nucleases, biosensors, and molecular switches.

5

Brief Description of the Drawings

FIGURE 1A is a Watson-Crick base pairing of the preferred α PNA module with a preferred single-stranded nucleic acid target.

FIGURE 1B is a preferred representation of an α PNA binding unit.

FIGURES 2 A-C show gel shift assay binding studies.

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FIGURE 3 is a chart showing circular dichroism (CD) intensity versus mole percent of the preferred α PNA.

FIGURE 4 is a chart showing CD intensity versus wavelength.

FIGURES 5 A-B are charts showing T_m versus calculated octanol- H_2O partition coefficients.

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FIGURE 6A shows a fluorescent image of a microtiter plate.

FIGURE 6B shows the quantification of hybridization as a function of fluorescence intensity.

Detailed Description of the Preferred Embodiments

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The present invention relates to the design, synthesis, and potential applications of peptide-based nucleic acid surrogates having a secondary structure. Preferably, the secondary structure of the peptide-based nucleic acid surrogate is α -helical with the structure known as α -helical peptide nucleic acids (α PNAs) which are new molecular constructs that merge the molecular recognition elements of nucleic acids with the predictable structural features of an α -helical peptide scaffold.

25

Peptide nucleic acids (PNAs) having a particular secondary structure are generally defined by the following subunit of $(AA^B\text{-}aa_n)_m$, wherein AA is any natural or unnatural (including but not limited to configurational

isomers) hydroxyl-containing amino acid including, but not limited to serine, threonine, homo-serine, etc.; aa is a natural or unnatural (including but not limited to configurational isomers) α -amino acid (including α -dialkylated α -amino acids); B is a natural nucleobase (i.e. B = adenine, cytosine, guanine, thymine, and uracil) or unnatural analog (ie. B = 7-deazapurines, 5-alkynylpyrimidines, "G-clamp", glycosylated 5-hydroxymethylpyrimidines, etc.); n is the number of natural or unnatural α -amino acids, wherein $n \geq 1$; and m is the number of repeating units, wherein wherein $m \geq 1$.

PNAs can possess different secondary structure elements (e.g., α -helix, 3_{10} helix, 2.2_7 ribbon, π helix, β ribbon, etc.) and are capable of sequence-specific binding to complementary single-stranded or double-stranded nucleic acid targets. A PNA can bind to complimentary double-stranded nucleic acid targets by strand invasion (i.e., partial denaturation of the double-stranded nucleic acid target) or by direct recognition of the duplex. Preferably, a PNA comprising an α -helical secondary structure (α PNA) is capable of sequence specific binding to single-stranded nucleic acid targets.

The preferred α PNA can also act as a functional α PNA, wherein the functional α PNA can be conjugated with various molecules and molecular systems, including but not limited to, fluorescent labeling tags, other peptides, particular molecules (e.g. biotin, etc.), DNA and RNA, etc.

FIGURE 1A shows a W-C (Watson-Crick) base pair binding between the preferred α PNA and complimentary single-stranded nucleic acid target, such as single-stranded DNA or RNA. FIGURE 1B shows a preferred structural definition for an α PNA. In the preferred structural definition, the preferred hydroxyl-containing amino acid bound to a nucleobase B is serine, although any hydroxyl-containing amino acid is suitable.

Forming and identifying PNAs having a particular secondary structure, such as α PNA, include the following steps: (1) devising a practical synthesis of PNAs having particular secondary structures that incorporate the

nucleoamino acid residue; and (2) purifying and characterizing of the resulting oligomeric PNAs having particular secondary structures; (3) evaluating of the ability of PNAs having particular secondary structures to bind to single-stranded nucleic acid; and (4) characterizing the structure of the resulting PNA•DNA complexes.

The synthesis of α PNA includes the following steps: (1) solid-phase peptide synthesis (SPPS) of an α PNA; and (2) cleaving the α PNA module from the resin with concomitant deprotection of all amino acid residues except cysteine to give a thiol-protected α PNA module. In addition, an optional third step of can be incorporated to link α PNA modules. Preferably, α PNA modules are linked by concomitant thiol deprotection and disulfide bond formation to produce a α PNA dimer, etc.

Protecting groups (PGs) are used to mask functional groups (FGs) that would interfere with, or not survive, a particular synthetic operation. A base-labile Fmoc PG is preferably chosen for the N-terminal amines. Acid-labile PGs, such as tert-butoxycarbonyl (Boc) and t-butyl ester, is preferably used for all sidechain functionalities except cysteine. For the cysteine residue, the I_2 -labile acetamidomethyl (Acm) PG is preferably chosen to facilitate a separate disulfide formation step. (Veber, D. F.; Milkowski, J. D.; Varga, S. L.; Denkwalter, R. G.; Hirschmann, R. *J. Am. Chem. Soc.* **1972**, *94*, 5456-5461). Pyrimidine-containing serine derivatives are prepared by I_2 -mediated nucleosidation of Fmoc-Ser(MTM)-OBn with silylated nucleobase. The acid-labile Rink amide linker (Rink amide linker = 4-(2',4'-dimethoxyphenylaminomethyl) phenoxyacetamido (Rink, H. *Tetrahedron Lett.* **1987**, *28*, 3787-3790)) is employed so that release of a fully extended peptide amide from the resin and removal of the acid-labile PGs could be achieved at the same time. Finally, DBU (DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene. Wade, J. D.; Bedford, J.; Sheppard, R. C. Tregear, G. W. *Pept. Res.* **1991**, *4*, 194) is used for Fmoc deprotection. Carpino's HATU (HATU = O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium

hexafluoro-phosphate. Carpino, L. A. *J. Am. Chem. Soc.* **1993**, *115*, 4397-4398)
reagent is used for all peptide couplings.

Either the manual or semi-automated SPPS of α PNAs can be
utilized and provide α PNAs in 10-15% overall yield from commercially available
5 Fmoc-Rink-MBHA resin after purification by gradient reverse-phase HPLC.

In order to form a disulfide linked α PNA dimer, I_2 -mediated Acn
deprotection/disulfide formation (Kamber, B.; Hartmann, A.; Eisler, K.; Riniker,
B.; Rink, H.; Sieber, P.; Rittel, W. *Helv. Chim. Acta* **1980** *63*, 899-915) of the
 α PNA monomers is utilized and symmetrical tail-to-tail α PNA dimers are formed
10 in good yield after HPLC purification.

Structure assignments for the α PNAs and α PNA dimers are
confirmed by any known procedure such as MALDI-TOF or ESI mass
spectrometry, and circular dichroism (CD) spectroscopy.

A further analyses of the production of α PNAs is disclosed in
15 Garner, P., Dey, S., Huang, Y., and Zhang, X., *Org. Lett.* Vol. 1, No. 3, 1999,
403-405, herein incorporated by reference.

α PNA modules with as few as five nucleobases can bind with high
affinity to complimentary single-stranded nucleic acid targets in a sequence-
specific manner. The ability of an α PNA to bind to a single-stranded nucleic acid
20 target can be evaluated via thermal denaturation and gel retardation
experiments. For a detailed explanation of binding affinities of α PNA to
complimentary nucleic acid targets, see Garner, P., Dey, S., and Huang, Y., *J.*
Am. Chem. Soc. **2000**, *122*, 2405-2406, herein incorporated by reference.

An important aspect of α PNA-DNA complexation is the preferred
25 directionality of binding, with the "parallel" (N/5') orientation favored both
thermodynamically (deduced from T_m data) and kinetically (deduced from
amount of hysteresis) over the "antiparallel" (N/3') orientation. This is analogous
to the well-known antiparallel (5'/3') strand orientation which is generally
observed in nucleic acid duplexes. The parallel complex Ac-

CTCCT•d(A₃GAGGAA₃) possesses different a secondary structure from the antiparallel counterpart Ac-CTCCT•d(A₃AGGAGA₃), which is evident from their CD profiles, particularly in the 230-290 nm region where the interaction of asymmetrically-disposed nucleobases is expected dominate the CD spectrum.

- 5 (Johnson, W. C., Jr. in *Circular Dichroism and the Conformational Analysis of Biomolecules*, Fasman, G. D. Ed. Plenum Press: New York, 1996). The spectrum of the complex formed from an α PNA with a symmetrical base sequence (Ac-CCTCC), capable of forming either a parallel or antiparallel complex, is also consistent with the parallel orientation. α PNAs may be N-
- 10 capped to obtain enhanced affinity and orientational specificity with complementary nucleic acid targets. For a detailed explanation of N-capped α PNAs, see Garner, P., Huang, Y, Dey, S. "Enhancement of α PNA Binding Affinity and Specificity via Hydrophobic Interactions" (unpublished).

- Binding studies show that changing the peptide backbone can
- 15 affect both the affinity and kinetics of α PNA binding to a nucleic acid target such as DNA. Combinatorial techniques are useful to explore the relationship between α PNA amino acid diversity and sequence-specific hybridization. The success of combinatorial techniques will depend largely on the development of an adequately sensitive and specific screen for hybridization. While there are
- 20 numerous examples of binding assays for nucleic acid and peptide libraries, (Pirrung, M. C. *Chem. Rev.* 1997,97, 473-488; Schultz, J. S. *Biotechnol. Prog.* 1996, 12, 729-743; Gold, L.; Polisky, b.; Uhlenbeck, o.; Yarus, M. *Annu. Rev. Biochem.* 1995, 64, 763-797; Pinilla, C.; Appel, J.; Blondelle, S.; Dooley, C.; Dörner, B.; Eichler, J.; Ostresh, J.; Houghten, R. A. *Biopolymers (Peptide*
- 25 *Science)* 1995, 37, 221-240; Geysen, H. M.; Meloen, R. H.; Barteling, S. J. *Proc. Natl. Acad. Sci. USA* 1984, 81, 3998-4002) relatively little work has been done on screening hybridization of nucleic acid surrogates. (King, D. J.; Ventura, D. A.; Brasier, A. R.; Gorenstein, D. G. *Biochemistry* 1998, 37, 16489-16493).

- "Immobilized nucleic acid hybridization" has become a common
- 30 technique in molecular biology. This assay is based on Watson-Crick base-

pairing and can be customized to screen α PNA libraries so that one can quickly identify compounds exhibiting high affinity towards immobilized complementary nucleic acid targets. Since only the structure of the peptide backbone is to be varied, the nucleobase sequence of the α PNA will remain fixed. Thus, the effect of α PNA structure variation on hybridization using a single immobilized oligonucleotide target can be evaluated. The α PNAs can be "tagged" with a suitable fluorophore and, after hybridization, the high-affinity α PNAs bound to the nucleic acid would give an easily-detected fluorescent signal. An analogous FISH experiment has been reported with fluorophore-tagged Nielsen PNAs conjugated to a solubility-enhancing amino acid. (Gildea, B. D.; Casey, S.; MacNiell, J.; Perry-O'Keef, H.; Sørensen, D.; Coull, J. M. *Tetrahedron Lett.* **1998**, 39, 7255-7258). Later, the "optimal" α PNA structure is selected from the initial pool of "hits" using a high-stringency assay like UV-melting.

A fluorescence based *in situ* hybridization assay for α PNAs has been developed. The results demonstrate that this analytical technique can be used to identify α PNAs that bind to DNA with high affinity and is suitable for screening α PNA libraries synthesized by combinatorial techniques.

Various fluorophores may be utilized in the fluorescence-based *in situ* hybridization assay for α PNA libraries. Preferably, the BODIPY (Aleshkov, S. B.; Fa, M.; Karolin, J.; Strandberg, L.; Johansson, L. B.-Å.; Wilczynska, M.; Ny, T. *J. Biol. Chem.* **1996**, 271, 21231-21238) fluorophore is used since it has no residual charge that may influence the hybridization of the α PNA and nucleic acid. Either thiol- or amine-reactive BODIPY dyes can be used depending on which stage the fluorophore is to be incorporated into the α PNA.

For a further discussion regarding hybridization studies, see Huang, Y., Garner, P. "Flourescence-Based *in situ* Hybridization (FISH) Assay for Screening α PNA Libraries" (unpublished).

The synthesis of purine-containing α PNAs is relatively difficult because of the ease with which acid-catalyzed depurination occurs. It was found

that a catalytic amount of ceric ammonium nitrate (CAN) (Hwu, J. R.; Jain, M. L.; Tsay, S.-C.; Hakimelahi, H. *Tetrahedron Lett.* **1996**, *37*, 2035-2038) can be used to remove the Boc group from the purine-containing modules under mild, neutral (oxidizing) conditions without depurination. These conditions will necessitate
 5 changing the cysteine protecting group (if one wishes to make disulfide-linked dimers) since the Ac^m group will also be susceptible to oxidative degradation. It is now possible to synthesize α PNAs that contain all 4 naturally-occurring nucleobases and study their hybridization properties.

The present invention is further illustrated by the following
 10 examples. It is to be understood that the present invention is not limited to the examples, and various changes and modifications may be made in the invention without departing from the spirit and scope thereof.

Examples

15 **Demonstration that α PNA binds to Complementary Single-Stranded DNA with High Affinity and in a Sequence Specific Manner**

The prototype α PNA design incorporated amino acid residues that were known to favor an α -helical formation:

backbone 1 (b1) = Ac-Cys^{Ac^m}-Gly-Ser^B-Asp-Ala-Glu-Ser^B-Ala₂-Lys-Ser^B-Ala₂-Glu-
 20 Ser^B-Ala-Aib-Ala-Ser^B-Lys-Gly-NH₂;
 backbone 1' (b1') = Ac-Aib-Gly-Ser^B-Asp-Ala-Glu-Ser^B-Ala₂-Lys-Ser^B-Ala-Glu-Ser^B-Ala-Aib-Ala-Ser^B-Lys-Cys^{Ac^m}-NH₂).

The α -helical secondary structure (~26%) of these backbone 1 α PNAs in the absence of DNA was confirmed by circular dichroism (CD) studies in H₂O. Both
 25 the N-terminal linked (b1) and C-terminal-linked (b1') cysteine dimers formed complexes with d(A₁₀) in H₂O (Table 1, entries 1 and 2). The latter complex had a T_m that was 12 °C higher than the former suggesting a thermodynamically preferred backbone orientation. The homothymine b1 α PNAs required a relatively long incubation period (up to 4 days at 4 °C until constant absorbance
 30 was reached) due to the slow kinetics of annealing. Accordingly, no

reassociation was observed during the cooling phase of the thermal denaturation experiments. By substituting cytosine (3 H-bonds per C•G base pair) for thymine (2 H-bonds per T•A base pair) in b1, even the monomeric C₅(b1) module was found to form a stable complex with d(TA₃G₅A₃T) in H₂O (entry 6). However, no binding was observed between these species in TE-buffer (10 mM Tris-HCl + 1 mM EDTA, pH 7.0) even after 4 days of incubation. For comparison, d(T₁₀)•d(A₁₀) had a T_m of 13 °C and d(C₅)•d(TA₃G₅A₃T) had a T_m of 19 °C under optimal DNA•DNA binding conditions (pH 7 buffer, 150 mM NaCl). These results suggested that both C₅(b1) and the T₅(b1)-dimers, which are expected to have net charges of -1 and -2 respectively at neutral pH, may be experiencing some charge-charge repulsion with the polyanionic DNA targets thus slowing down the rate of hybridization.

In order to accelerate the rate of hybridization of αPNAs and single-stranded DNA, a new αPNA backbone was designed in which the negatively charged Asp and Glu residues were replaced with Lys:

backbone 2 (b2) = Ac-Cys^{Ac}-Lys-(Ser^B-Ala₂-Lys)₄-Ser^B-Gly-Lys-NH₂;

backbone 2' (b2') = Ac-Lys₂-(Ser^B-Ala₂-Lys)₄-Ser^B-Gly-Cys^{Ac}-NH₂.

This modification resulted in αPNAs having a net positive charge at neutral pH and was expected to enhance nonspecific binding to negatively charged DNA prior to sequence-specific base-pairing. (Cf. Corey, D. R. *J. Am. Chem. Soc.* 1995, 117, 9373-9374). Thus, both the T₅(b2)- and T₅(b2')-dimers formed complexes that exhibited well-defined melting curves in TE-buffer after only an overnight incubation period (entries 3 and 4) and, unlike b1 and b1', did not show a significant difference in stability. Even the T₅(b2) module hybridized with d(A₁₀) – albeit weakly as expected for a complex held together by only A•T base pairs (entry 5). Also, b2 αPNAs that contained cytosine resulted in remarkably stable αPNA•DNA complexes. For example, C₅(b2)•d(TA₃G₅A₃T) exhibited a T_m of 54 °C in TE-buffer and no hysteresis was observed in the cooling curve. This implies that equilibration is being achieved during the cooling cycle. This T_m is 20 °C higher than that of C₅(b1)•d(TA₃G₅A₃T) in H₂O (compare entries 6 and 7) and

35 °C higher than the corresponding DNA-DNA duplex (in TE-buffer + 150 mM NaCl). Indeed, faster and stronger hybridization was observed for all matched cytosine-containing complexes formed with b2 α PNAs and complementary single-stranded DNAs with equilibrium dissociation constants (K_d) in the micromolar range. (Marky, L. A.; Breslauer, K. J. *Biopolymers* 1987, 26, 1601-1620). Added salt was expected to disrupt favorable charge-charge interactions between cationic α PNAs and anionic DNA. Accordingly, while $C_5(b2) \cdot d(TA_3G_5A_3T)$ did exhibit a T_m of 49 °C in TE-buffer + 150 mM NaCl, no reassociation was observed during the cooling run. The presence of flanking bases on the DNA target strand also resulted in complexes with higher T_m (compare entries 7 and 8), as well as greater hypochromicity and less hysteresis. Incorporation of mismatches into the α PNA (entries 7 and 10) resulted in a marked lowering of T_m for the complexes, a result which is consistent with sequence-specific base recognition. Furthermore, the parallel orientation (N-terminus of α PNA adjacent to the 5'-end of DNA or N/5') resulted in a higher T_m when compared to the antiparallel alternative (entries 11 and 12). The melted antiparallel (N/3') complex did not reassociate during the cooling cycle indicating slower binding kinetics relative to the parallel orientation.

Table 1

entry	α PNA (N→C)	DNA sequence (5'→3')	T _m (°C)
1.	T ₅ (b1)-dimer	d(A ₁₀)	39
2.	T ₅ (b1')-dimer	d(A ₁₀)	51
3.	T ₅ (b2)-dimer	d(A ₁₀)	46, 54 ^a
4.	T ₅ (b2')-dimer	d(A ₁₀)	46, 57 ^a
5.	T ₅ (b2)	d(A ₁₀)	17
6.	C ₅ (b1)	d(TA ₃ G ₅ A ₃ T)	34
7.	C ₅ (b2)	d(TA ₃ G ₅ A ₃ T)	54
8.	C ₅ (b2)	d(G ₅)	35
9.	CCTCC(b2)	d(A ₃ GGAGGA ₃)	49
10.	CCTCC(b2)	d(TA ₃ G ₅ A ₃ T)	38
11.	CTCCT(b2)	d(A ₃ AGGAGA ₃)	32
12.	CTCCT(b2)	d(A ₃ GAGGAA ₃)	37

^aTwo-step melting was observed.

FIGURE 2 shows gel shift binding studies on (A) CCTCC(b2) + d(A₃GGAGGA₃), (B) CTCCT(b2) + d(A₃GAGGAA₃) (lanes 1-4) and CTCCT(b2) + d(A₃AGGAGA₃) (lanes 5-8), and (C) T₅(b2)-dimer + d(C₃T(TC₂)₂A₁₀C(TC₂)₃). Solutions were made up by combining DNA (40 μM) with varying amounts of αPNA in 7.5 μL of TE-buffer. Before electrophoresis, 2.5 μL of loading buffer (0.01% xylene cyanol FF, 0.01% bromophenol blue solution, 60% (w/v) glycerol in running buffer) was added to the sample, mixed, and 3.5 μL of the sample was loaded onto the gel. Free DNA and αPNA-bound DNA were resolved by nondenaturing 14% PAGE in 44 mM Tris-borate, pH 7.2 for 1 hour at 14 V cm⁻¹ at 4 °C. The ratios of αPNA to DNA in (A) for lanes 1-8 are 0/1, 1/2, 3/4, 1/1, 5/4, 3/2, 7/4 and 2/1. Lane 9 represents only αPNA. The ratios in (B) for lanes 1-4 as well as 5-8 are 0/1, 1/1, 2/3, and 4/7. The ratios in (C) from lanes 1-8 are 0/1, 1/2, 1/1, 3/2, 2/1, 5/2, 3/1, and 4/1. Gels were developed using the PlusOne™ DNA silver staining kit (Pharmacia Biotech).

To investigate the stability of the α PNA-DNA hybrids as well as their binding stoichiometry and structure, both gel-shift mobility and circular dichroism (CD) titration studies were performed. For the complex between CCTCC(b2) and d(A₃GGAGGA₃), a single new slower-migrating species corresponding to CCTCC(b2)•d(A₃GGAGGA₃) was detected in the gel shift assay as shown in FIGURE 2A. The intensity of the d(A₃GGAGGA₃) band steadily decreased with increasing amounts of CCTCC(b2) until there was no unbound single-stranded DNA at an α PNA/DNA ratio of 1.5. The detection of a triplex between two CCTCC(b2)'s (net charge +6 each) and d(A₃GGAGGA₃) (net charge -10) is precluded on the grounds that the resultant ternary complex would have a net positive charge and therefore be expected to migrate towards the negative electrode. Evidence for duplex formation was also obtained from experiments on CTCCT(b2) + d(A₃GAGGAA₃) and CTCCT(b2) + d(A₃AGGAGA₃) as shown in FIGURE 2B. These gels also confirmed that the parallel (N/3') orientation resulted in stronger binding. No binding was observed between an "abasic" α PNA(b2) peptide Ac-Cys^{Ac}-Lys-(Ser-Ala₂-Lys)₄-Ser-Gly-Lys-NH₂ and d(TA₃G₅A₃T), which underscores the role that nucleobases play in α PNA molecular recognition. The concentration of this "abasic" α PNA(b2) was determined by quantitative ninhydrin analysis (Sarin, V. K.; Kent, S. B. H.; Tam, J. P.; Merrifield, R. B. *Anal. Biochem.*, **1981**, 117, 147-157). Interestingly, the PAGE experiment with T₅(b2)-dimer (net charge +12) and d(C₃T(TC₂)₂A₁₀C(TC₂)₃) (net charge -29) produced an additional slower-moving species at the expense of the initially-formed species at α PNA/DNA ratios greater than 1 as shown in FIGURE 2C. This result is consistent with the formation of a ternary complex.

FIGURE 3 shows job plots for CDs of C₅(b2) + d(TA₃G₅A₃T) (at 258 nm, squares), CCTCC(b2) + d(A₃GGAGGA₃) (at 258 nm, circles), and T₅(b2)-dimer + d(A₁₀) (at 261 nm, triangles). Spectra were recorded at 5 °C using a JASCO J-600 CD spectro-polarimeter. Samples having a total concentration ([α PNA] + [DNA]) of 12 μ M were made up in doubly deionized water and placed

in a stoppered optical quartz cell (1 cm pathlength). Dry air was purged through the sample compartment. Each data point represents the average of eight (baseline corrected) points. FIGURE 3 shows further support for the binding stoichiometries of the α PNA•DNA complexes comes from CD titration studies on
5 $C_5(b2) + d(TA_3G_5A_3T)$ and $CCTCC(b2) + d(A_3GGAGGA_3)$, which both showed intensity minima at 50 mol% α PNA as expected for a 1:1 binding stoichiometry. The inset of FIGURE 3 shows that in addition to a duplex, $T_5(b2)$ -dimer + $d(A_{10})$ also showed evidence of a 2:1 complex. This is in line with the PAGE experiment with $T_5(b2)$ -dimer and $d(C_3T(TC_2)_2A_{10}C(TC_2)_3)$.

10 FIGURE 4 shows the comparative CD spectra of (a) DNA $d(A_3GGAGGA_3)$ alone, (b) α PNA $CCTCC(b2)$ alone, and (c) a 1:1 mixture of $d(A_3GGAGGA_3) + CCTCC(b2)$ (6 μ M each) in distilled H_2O . Spectra were recorded as described in FIGURE 3. In FIGURE 4, the CD spectrum of a solution containing equimolar amounts of $CCTCC(b2)$ and $d(A_3GGAGGA_3)$ shows the
15 characteristic CD signatures of both a peptide α -helix (minima at 220 and 206 nm, maximum at 196 nm) (Woody, R. M. *Methods Enzymol.* 1995, 246, 34-71) and a DNA B-helix (maximum at 280 and minimum at 255 nm). (Gray, D. M.; Ratliff, R. L.; Vaughan, M. R. *Methods Enzymol.* 1992, 211, 389-406). Since control CD spectra show that the individual b2 α PNA and DNA components are
20 essentially disordered in the absence of the other, it appears that they are each acting as templates for hybridization. Analogous behavior has been noted previously for peptides corresponding to the basic regions of DNA-binding proteins. (Patel, L.; Abate, C.; Curran, T. *Nature* 1990, 347, 572-575; Weiss, M. A.; Ellenberger, T.; Wobbe, C. R.; Lee, J. P.; Harrison, S. C.; Struhl, K. *Ibid.*,
25 1990, 347, 575-578; Talanian, R. V.; McKnight, C. J.; Kim, P. S. *Science* 1990, 249, 769-771).

Binding Affinity and Orientational Specificity Modulated by Hydrophobic N-Caps

The binding properties of a series of N-capped α PNAs that incorporated additional α -stacking interactions were examined. This experiment was based on the assumption that tighter binding would result if "end-fraying" of α PNAs were minimized by incorporating such interactions at one or both ends of the α PNA chain. Such an effect could be important since the described α PNA modules rely on only 5 base-pair interactions and end-fraying could adversely affect up to 50% (in CTTTC) of the interstrand Watson-Crick hydrogen-bonds. The enhancement of duplex stability through such α - α interactions had been demonstrated with unpaired "dangling" nucleobases at the 5'-ends of RNA, (M. Petersheim, D. H. Turner, *Biochemistry*, 1983, 22, 256-263) and analogous effects resulted when nucleobases as well as their unnatural aromatic counterparts were incorporated at the 5'-ends of DNA. (M. Senior, R. A. Jones, K. J. Breslauer *Biochemistry*, 1988, 27, 3879-3885; K. M. Guckian, B. A. Schweitzer, R. X.-F. Ren, C. J. Sheils, D. C. Tahmassebi, E. T. Kool, *J. Am. Chem. Soc.*, 2000, 122, 2213-2222).

It was found that N-capped α PNAs lead to both enhanced affinity as well as orientational specificity with complementary ssDNA targets. Unexpectedly, the observed N-cap effects in the preferred parallel series were found to be better correlated to hydrophobicity rather than π -stacking.

N-capped α PNAs were synthesized via the SPPS protocol but substituting an appropriate carboxylic acid in the N-capping step. All of the α PNAs possess the backbone shown in FIGURE 1B and are described by the N-cap followed by the nucleobase sequence (N- to C-terminus from left to right). Electrospray ionization mass spectral (ESI⁺-MS) characterization data for N-capped α PNAs: 4-phenylbutyryl-CTCCT calcd 2811.41, found 2812.61 \pm 0.41; 4-(p-methoxyphenyl)butyryl-CTCCT calcd 2841.42, found 2842.36 \pm 0.13; 4-(p-nitrophenyl)butyryl-CTCCT calcd 2856.40, found 2857.55 \pm 0.24; 4-(2-naphthyl)butyryl-CTCCT calcd 2861.43, found 2862.68 \pm 0.40; 4-(1-pyrenyl)butyryl-CTCCT calcd 2935.44, found 2937.02 \pm 0.39; n-butyryl-CTCCT

calcd 2735.38, found 2736.52±0.54; n-hexanoyl-CTCCT calcd 2763.41, found 2766.01±1.19; 3-cyclopentylpropionyl-CTCCT calcd 2789.43 found 2792.10±1.01; 4-cyclohexylbutyryl-CTCCT calcd 2817.46, found 2818.41±0.74; 4-cyclohexylbutyryl-CCTCC calcd 2802.46, found 2803.12±0.39; 4-cyclohexylbutyryl-CCCCC calcd 2787.46, found 2787.83±0.06.

The N-caps investigated consisted of a series of arenyl and aliphatic carboxylic acids. Both sets of N-caps included a flexible methylene linker to allow for the anticipated cap-base interaction without significantly disrupting the complex structure. Thermal UV denaturation (T_m) data for the equilibria between N-cap-CTCCT and d(A₃GAGGAA₃) (N/5' or parallel orientation) and N-cap-CTCCT and d(A₃AGGAGA₃) (N/3' or antiparallel orientation) are collected in Table 2. Thermal UV denaturation curves were obtained with sample solutions made by combining the N-capped α PNA and ssDNA components (5 μ M each) in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7). These solutions were then incubated overnight at 4 °C.

TABLE 2

entry	cap-CTCCT	d(A ₃ GAGGA-A ₃) T_m (heating, cooling)	d(A ₃ AGGAG-A ₃) T_m (heating) ^a
1.	cap = CH ₃ CO	37.0, 32.0	32.0
2.	CH ₃ CH ₂ CH ₂ CO	40.0, 36.7	30.2
3.	CH ₃ CH ₂ CH ₂ CH ₂ CH ₂ CO	41.0, 35.0	33.4
4.	C ₅ H ₉ CH ₂ CH ₂ CO	43.7, 38.2	33.8
5.	C ₆ H ₁₁ CH ₂ CH ₂ CH ₂ CO	47.9, 43.7	33.4
6.	C ₆ H ₅ (CH ₂) ₃ CO	41.8, 38.5	33.6
7.	p-MeOC ₆ H ₄ (CH ₂) ₃ CO	41.3, 37.0	34.2
8.	p-NO ₂ C ₆ H ₄ (CH ₂) ₃ CO	41.6, 37.8	34.4
9.	2-naphthyl(CH ₂) ₃ CO	42.7, 40.0	36.3
10.	1-pyrenyl(CH ₂) ₃ CO	42.6, 43.8	39.0

^aNo cooperative transition was observed during the cooling cycle.

A number of interesting trends emerge from Table 2. First, all of the N-cap modifications resulted in higher T_m values for the parallel α PNA•DNA complexes relative to the "parent" N-acetylated system. Reduced hysteresis was observed for all parallel complexes during the cooling cycle which was indicative of faster annealing kinetics. However, contrary to our expectations, the most striking enhancement occurred in the aliphatic N-cap series, with the 4-(cyclohexyl)butyryl-N-cap resulting in the highest T_m (heating, cooling) values (47.9, 43.7 °C). This represented an 11-12 °C increase compared to the parent N-acetylated α PNA. Such behavior would not be expected if π -stacking was the primary means of stabilizing these α PNA•DNA complexes. The relative insensitivity of 4-(phenyl)butyryl N-cap melting temperatures to electron-donating and electron-withdrawing phenyl substituents is consistent with this interpretation. (Cf. F. Cozzi, M. Cinquini, R. Annuziata, J. S. Siegel, *J. Am. Chem. Soc.* **1993**, *115*, 5330-5331).

Table 3 shows analogous T_m enhancements with the symmetrical sequences N-4-(cyclohexyl)butyryl-CCTCC•d(A₃GGAGGA₃) (60.8, 51 °C, entry 1) and N-4-(cyclohexyl)butyryl-CCCCC•d(TA₃GGGGGA₃T) (64.9, 66 °C, entry 2), thus excluding the possibility that these trends were related solely to the base sequence. The incorporation of a single base-pair mismatch (N-4-(cyclohexyl)butyryl-CCTCC•d(TA₃GGGGGA₃T)) lowered the T_m (heating, cooling) of the complex by 15 and 10 °C respectively (entry 3), which is consistent with our previous observations in the N-acetyl series and indicates that the N-cap effect is decoupled from base-pairing. This reduction in melting temperature presumably arises from residual hydration of the mismatched nucleobases and provides additional support for our proposed Watson-Crick binding model.

Table 3

entry	α PNA•DNA complex	T_m (heating, cooling)
1.	C ₆ H ₁₁ (CH ₂) ₃ CO-CCTCC 5' d(A ₃ -GGAGG-A ₃) 3'	60, 51
2.	C ₆ H ₁₁ (CH ₂) ₃ CO-CCCCC 5' d(TA ₃ -GGGGG-A ₃ T) 3'	65, 66
3.	C ₆ H ₁₁ (CH ₂) ₃ CO-CC T CC 5' d(TA ₃ -GG G GG-A ₃ T) 3'	50, 46

FIGURE 5 shows a plot of T_m versus calculated octanol-H₂O partition coefficients (log P values) for each N-cap. FIGURE 5 shows that the melting temperatures of α PNA•DNA complexes is proportional to N-cap hydrophobicity. (A. Leo, C. Hansch, D. Elkins, *Chem. Rev.* **1971**, *71*, 525-616). It is likely that the strong dependence of T_m on log P in the parallel aliphatic N-cap series is due to a combination of "classical" (entropically-driven) desolvation and "nonclassical" (enthalpically-driven) packing hydrophobic effects. (For a succinct introduction to the hydrophobic effect and its biological implications, see: A. Fersht, *Structure and Mechanism in Protein Science*; W. H. Freeman, New York, **1999**, Chapter 11). For the antiparallel complexes, which exhibit uniformly lower T_m values than their parallel counterparts, a weaker dependence is found for the arenyl N-caps. This suggests the existence of compensating π -stacking effects superimposed on the hydrophobic effects and is probably related to different overall structures (see CD data), but this is still conjecture that will have to await the completion of further α PNA•DNA structural studies. On the other hand, melting temperature is essentially independent of N-cap hydrophobicity in both the parallel-aromatic and antiparallel-aliphatic series. By demonstrating that strategically placed aliphatic groups can be used to modulate the affinity and orientational specificity of α PNA complexation to DNA, the

repertoire of binding interactions available to these novel nucleic acid surrogates has been expanded. Others have noted similar stabilizing effects on oligonucleotide duplexes that were 5'-capped with aliphatic steroids. ((a) R. L. Letsinger, G. Zhang, D. K. Sun, T. Ikeuchi, P. S. Sarin, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6553-6556. (b) S. M. Gryaznov, D. H. Loyd, *Nucleic Acid Res.* **1993**, *21*, 5909-5915. (c) C. F. Bleczynski, C. Richert, *J. Am. Chem. Soc.* **1999**, *121*, 10889-10894). However, our work provides a direct comparison between aliphatic and aromatic end-caps showing that - at least for α PNAs - aliphatic end-caps can have a stronger effect on hybridization than aromatic end-caps.

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Assay for Determining Hybridization of α PNA and Single-Stranded Nucleic Acid Targets

FIGURE 6 shows a preferred hybridization of fluorescence-labeled α PNAs with gel-immobilized DNA d(A₄GGAGGA₄) on microtiter plate. A1-A2, 5 μ M BODIPY[®] FLIA labeled abasic peptide; A3-A5, deionized water; B1-B5, 5 μ M F-CCTCC; C1-C5, 10 μ M F-CCTCC. a) Fluorescent image of the plate (FluorImager SI, Molecular Dynamics). b) Quantification of hybridization as a function of fluorescence intensity as determined with a FluorMax Plate Reader. Excitation wavelength: 480 nm; Emission wavelength: 512 nm. The ratio of the average intensity of C1-C5 to B1-B5 is 2 to 1 after subtracting the background A1-A5 signal.

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Since the synthesized α PNAs incorporate a terminal Ac^m-protected cysteine residue, the thiol-reactive dye, BODIPY[®] FLIA, is introduced to Ac-Cys^{Ac^m}-Lys-(Ser^C-Ala₂-Lys)₂-Ser^T-(Ala₂-Lys-Ser^C)-Gly-Lys-NH₂ (CCTCC) after SPPS. The course of the labeling reaction can be easily followed by analytical HPLC and only a single HPLC purification is needed for the whole process. Based on comparative UV, CD, and PAGE experiments conducted with both labeled and unlabeled CCTCC and d(A₃GGAGGA₃), it was concluded that BODIPY has little effect on the complex stability or conformation and is thus suitable for *in situ* hybridization assays. Commercially obtained oligonucleotide

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d(A₃GGAGGA₃) containing a 5'-terminal acrylamide groups was immobilized on a 96 well microtiter plate following the procedure developed by Mosaic Technologies. (Rehman, F. N.; Audeh, M.; Abrams, E. S.; Hammond, P. W.; Kenney, M.; Boles, T. C. *Nucleic Acids Res.*, 1999, 27, 649-655).

5 After immobilization, a standard silver stain was applied and the dark color obtained proved that the acryldite DNA was immobilized on the gel. The spots may also be quantified by a gel documentation system. As a control, BODIPY labeled abasic peptide with unmodified Ser residues was synthesized as well. The results shown in FIGURE1 shows that α PNA binding to the
10 immobilized DNA is not a consequence of the fluorescence tag. The signal that the FluroMax Reader and FluorImager SI detected was all due to the Watson-Crick base-pairing. Only the F-CCTCC shows the particular fluorescence emission spectra of the BODIPY dye (data not shown). If we consider the readings for F-labeled abasic peptide and water as background, the ratio of the
15 average fluorescence intensity only depends on the concentration of the α PNA. Since the concentration of immobilized DNA is larger than that of added α PNA, this correlation shows that the hybridization assay is quantitative for bound α PNA.

20 The invention has been described with reference to the preferred embodiment. Obviously, modifications and alterations will occur to others upon reading and understanding of the preceding detailed description. It is intended that the invention be construed as including all such alterations and modifications insofar as they come within the scope of the appended claims or equivalents thereof.

Having thus described the invention, it is claimed:

1. A peptide-based nucleic acid surrogate comprising:
a secondary structure; and
a $(AA^B-aa_n)_m$ subunit, wherein AA is a hydroxyl-amino acid, B is a
5 nucleobase, aa is an amino acid, $n \geq 1$, and $m \geq 1$.
2. The peptide-based nucleic acid surrogate of claim 1, wherein the
secondary structure is an alpha-helical structure.
- 10 3. The peptide-based nucleic acid surrogate of claim 1, wherein B is
thymine, cytosine, adenine, guanine, and uracil.
4. The peptide-based nucleic acid surrogate of claim 1, wherein $m \geq 5$.
- 15 5. The peptide-based nucleic acid surrogate of claim 1, wherein the
surrogate further comprises a protecting group.
6. The peptide-based nucleic acid surrogate of claim 5, wherein the
protecting group is a base-labile fluorenyl methoxycarbonyl protecting
20 group for an N-terminus amine of the peptide-based nucleic acid
surrogate.
7. The peptide-based nucleic acid surrogate of claim 5, wherein the
protecting group is an I_2 -labile acetamidomethyl protecting group for the
25 cysteine side chain amino acid.
8. The peptide-based nucleic acid surrogate of claim 1, wherein B is a purine
nucleobase selected from the group consisting of adenine, 6-chloro-2-
aminopurine and guanine.

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9. The peptide-based nucleic acid surrogate of claim 9, wherein the purine nucleobase further comprises an acid-labile tert-butoxycarbonyl protecting group.
- 5 10. The peptide-based nucleic acid surrogate of claim 1, wherein the surrogate selectively binds with a nucleic acid target.
11. The peptide-based nucleic acid surrogate of claim 1, wherein an N-terminus of the surrogate comprises a carboxylic acid.
- 10 12. A peptide-based nucleic acid surrogate comprising:
an alpha-helical secondary structure;
a protecting group; and
a $(AA^B-aa_n)_m$ subunit, wherein AA is a hydroxyl-amino acid, B is a
15 nucleobase, aa is an amino acid, $n \geq 1$, and $m \geq 1$.
13. The peptide-based nucleic acid surrogate of claim 12, wherein B is thymine, cytosine, adenine, guanine, and uracil.
- 20 14. The peptide-based nucleic acid surrogate of claim 12, wherein $m \geq 5$.
15. The peptide-based nucleic acid surrogate of claim 12, wherein the protecting group is a base-labile fluorenyl methoxycarbonyl protecting group for an N-terminus amine of the peptide-based nucleic acid
25 surrogate.
16. The peptide-based nucleic acid surrogate of claim 12, wherein the protecting group is an I_2 -labile acetamidomethyl protecting group for the cysteine side chain amino acid.
- 30

17. The peptide-based nucleic acid surrogate of claim 12, wherein B is a purine nucleobase selected from the group consisting of adenine, 6-chloro-2-aminopurine and guanine.
- 5 18. The peptide-based nucleic acid surrogate of claim 17, wherein the purine nucleobase further comprises an acid-labile tert-butoxycarbonyl protecting group.
- 10 19. A method of forming a peptide-based nucleic acid surrogate having a secondary structure comprising the steps of:
solid-phase peptide synthesis of a resin-bound peptide-based nucleic acid surrogate; and
cleaving the resin from the peptide-based nucleic acid surrogate.
- 15 20. The method of claim 19, further comprising the steps of:
thiol deprotection of the peptide-based nucleic acid surrogate; and
disulfide bonding of two peptide-based nucleic acid surrogates having a secondary structure to form a dimer.
- 20 21. The method of claim 19, wherein the secondary structure is an alpha-helical structure.
22. The method according to claim 19, further comprising the step of:
hybridizing the peptide-based nucleic acid surrogate with a nucleic acid
25 target; and
screening the hybridization of the peptide based nucleic acid surrogate with a single-stranded nucleic acid target.
23. The method according to claim 19, further comprising the step of:

capping an N-terminus of the peptide-based nucleic acid surrogate with a carboxylic acid.

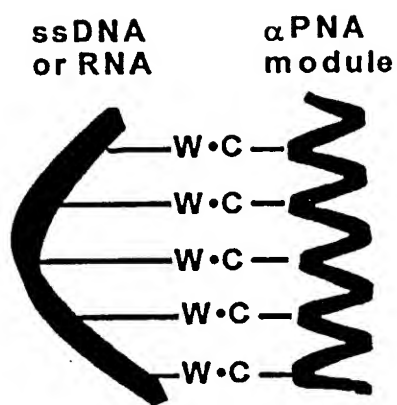
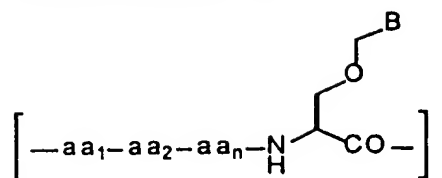


FIGURE 1A

α PNA
repeating unit:



aa = amino acid

B = nucleobase

W•C = Watson-Crick base pair

FIGURE 1B

FIGURE 2A



FIGURE 2B

1 2 3 4 5 6 7 8



FIGURE 2C

1 2 3 4 5 6 7 8



FIGURE 3

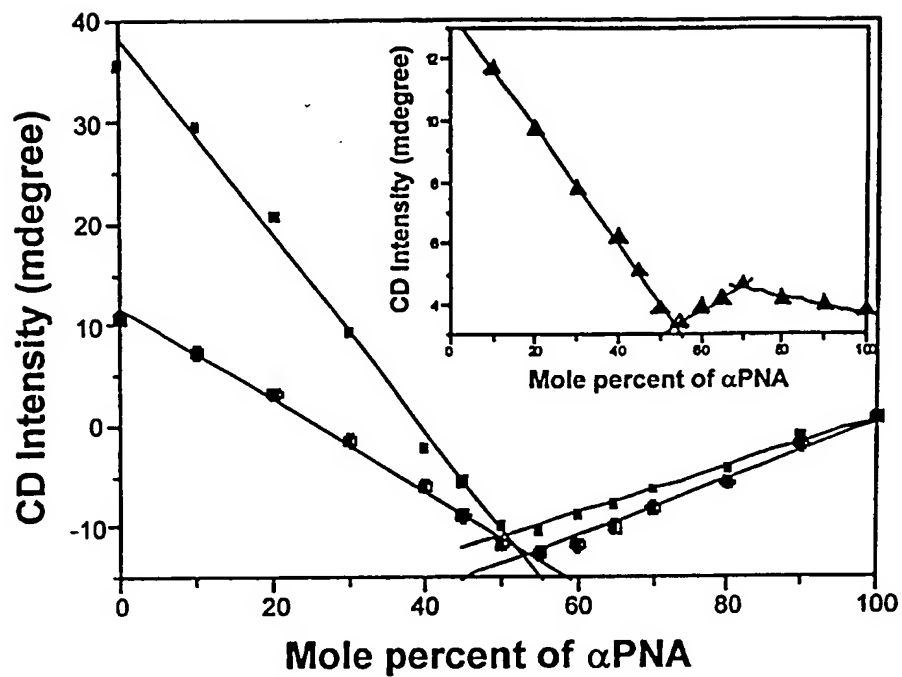


FIGURE 4

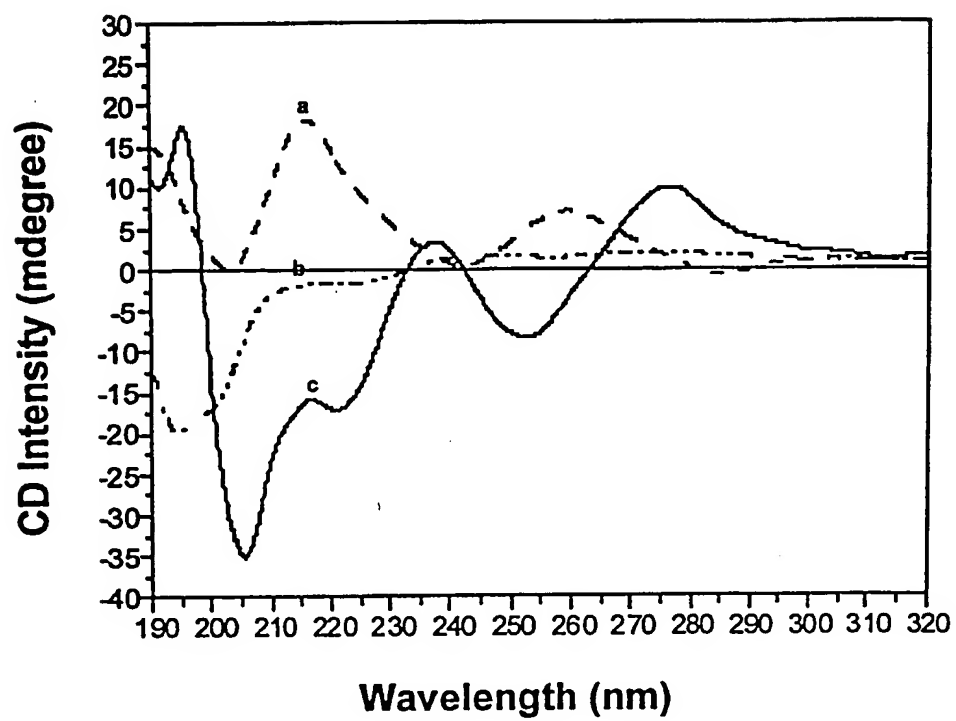


FIGURE 5A

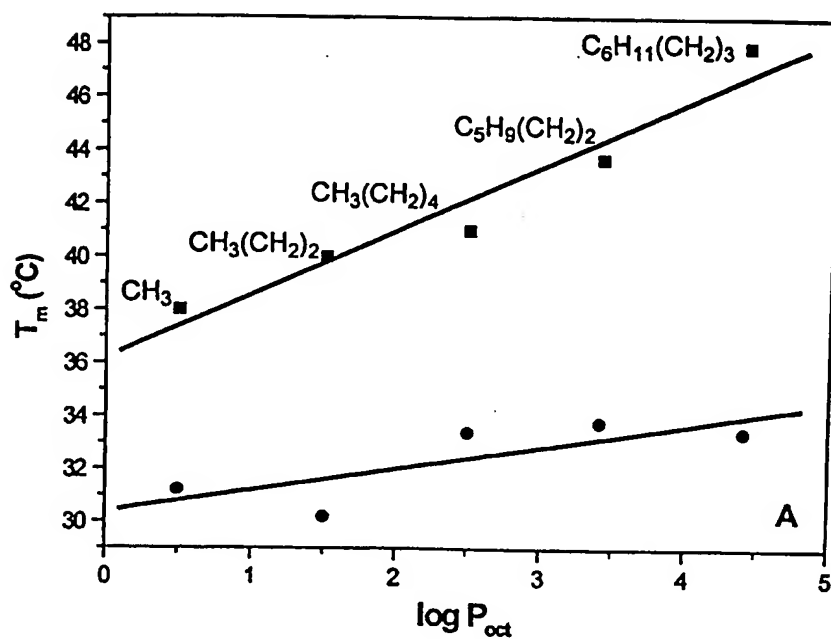
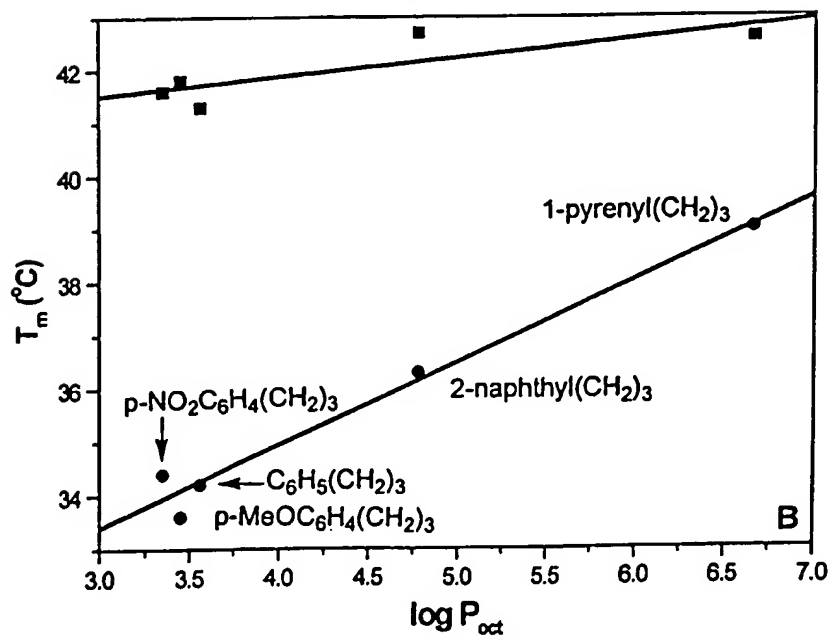


FIGURE 5B



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FIGURE 6A

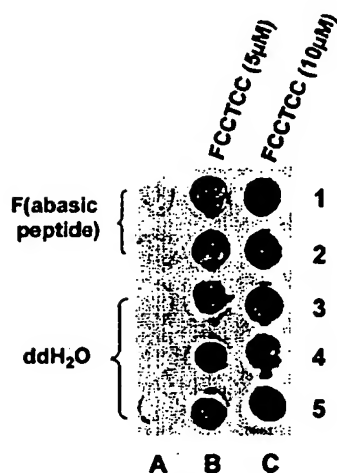
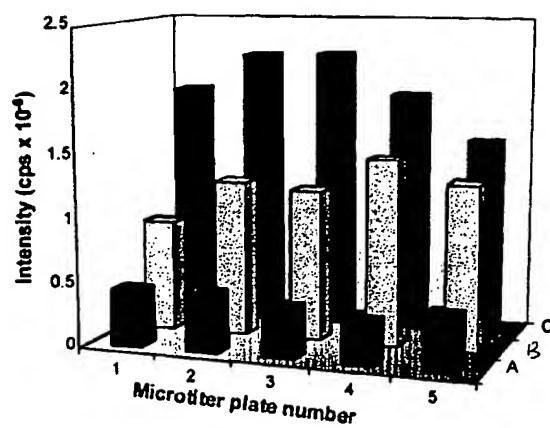


FIGURE 6B



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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US00/21845

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07H 19/00, 21/00, 21/02, 21/04

US CL : 536/22.1, 23.1, 24.3, 25.3, 25.32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22.1, 23.1, 24.3, 25.3, 25.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN

search terms: PNA, peptide nucleic acid, synthesis, protective groups, hybridization

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	WO 92/20702 A1 (BUCHARDT et al) 26 November 1992, see entire document.	1, 3-6, 8-11 ----- 2, 7, 12-23
X - Y	US 5,705,333 A (SHAH et al) 06 January 1998, see entire document.	1-6, 8-15, 17, 18 ----- 19-23

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	*T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"O"	document referring to an oral disclosure, use, exhibition or other means	"A" document member of the same patent family
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Date of the actual completion of the international search

25 SEPTEMBER 2000

Date of mailing of the international search report

17 OCT 2000

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